In vivo Incorporation of Labeled Methionine into Proteins, Vitellogenin, and Vitellin in Females of the Penaeid Shrimp *Penaeus semisulcatus* de Haan

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Abstract. [^{35}S]-Methionine was injected into 11 intact vitellogenic females of the penaeid shrimp *Penaeus semisulcatus* de Haan. Levels of radiolabeled methionine, total protein and vitellogenin/vitellin (Vg/Vt) were measured in the hemolymph during 24 h following methionine injections. The same parameters were measured 24 h after injection, in the hepatopancreas and ovaries of sacrificed females. Proteins were precipitated by trichloroacetic acid and Vg/Vt was immunoprecipitated by anti-Vt serum.

Hemolymphatic protein and Vg/Vt levels were constant throughout the 24 h of the experiment starting from the first sampling of hemolymph, two h after injection. Similar amounts of Vg/Vt were found in the hepatopancreas compared to the ovary 24 h after injection, and 10% of the labeled protein in the ovary and 6.4% in the hepatopancreas were Vg/Vt. Free-labeled methionine was still present in all tissues examined after 24 h. The labeled protein and Vg/Vt in the ovary and the hepatopancreas could not be explained by the hemolymphatic content of the two organs. The hemolymphatic Vg is 24–fold labeled over the ovarian Vt, 24 h after injection. The results indicate more intense involvement of the hepatopancreas in the vitellogenic process than can be deduced from earlier *in vitro* studies. This study confirms the earlier definition of the oogenic stage of rapid Vt accumulation in the ovary (AOD range of 150–250 μm) and also indicates a role for the hemolymph in transporting Vg between its processing sites.

Introduction

Crustacean vitellin (Vt) is a lipoglycoprotein that accumulates in the oocyte cytoplasm. Vt is used later for the nutrition of the non-feeding embryo and larval stages. Vitellogenin (Vg), a vitellin-immunoidentical protein, is a female-specific protein found in crustacean extraoocytic tissues and hemolymph (Meusy and Payen, 1988). Vg is produced by the subepidermal adipose tissue (SAT) of anostracan (Van Beek *et al.*, 1987), isopod (Picaud, 1980; Souty and Picaud, 1981; Picaud *et al.*, 1989), and amphipod (Junera and Croisille, 1980; Croisille and Junera, 1980) crustaceans before it is transported by the hemolymph into the developing oocytes.

Decapod Vg is present in the SAT (Meusy *et al.*, 1983; Vazquez-Boucard, 1985; Tom *et al.*, 1987), hepatopancreas (Paulus and Laufer, 1987; Quackenbush and Keeley, 1988; Quackenbush, 1989a, b; Fainzilber *et al.*, 1992) and hemolymph (Kerr, 1969; Cecalldi, 1970; Wolin *et al.*, 1973; Fyffe and O'Connor, 1974; Caubere *et al.*, 1976; Dehn *et al.*, 1983; Durliat, 1984; Marzari *et al.*, 1986; Tom *et al.*, 1987; Yano, 1987; Quackenbush, 1989a; Shafir *et al.*, in prep.). A major source of decapod yolk is Vt synthesis by the ovary itself (Lui *et al.*, 1974; Lui and O'Connor, 1976, 1977; Dehn *et al.*, 1983; Eastman-Reks and Fingerman, 1985; Yano and Chinzei, 1987; Quackenbush, 1989a; Browdy *et al.*, 1990). The site of Vt synthesis in *Penaeus japonicus* was suggested to be the follicle cells (Yano and Chinzei, 1987). No Vg synthesis occurred in *in vitro* incubated SAT of *Penaeus semisulcatus* (Fainzilber *et al.*, 1992); however, three studies have reported Vg synthesis in the decapod hepatopancreas: Quackenbush and Keeley (1988) in *Uca pugilator*; Quackenbush (1989a) in *Penaeus vannamei* and Fainzilber *et al.* (1992) in *Penaeus semisulcatus*.

Vt endocytosis by the ovaries of several decapod species, including penaeids has been reported by Beams and Kessel, 1963; Hinsch and Cone, 1969; Wolin *et al.*, 1973; Duronslet *et al.*, 1975; Zerbib, 1979; Schade and Shivers, 1980; Zerbib and Mustel, 1984; and Jugan and Soyez, 1985. Vg receptors were found and isolated from the oocytic cytoplasm membrane of two decapod species (Jugan, 1985; Laverdure and Soyez, 1988; Jugan and Van Herp, 1989).

The penaeid ovary is a well established major synthetic site of Vt compared to the minor quantitative contribution of the penaeid hepatopancreas to Vg production, as was revealed by *in vitro* incubations of penaeid hepatopancreas and ovaries in the presence of labeled amino acids. Yano and Chinzei (1987) found no Vg synthesis at all in *P. japonicus* hepatopancreas in contrast to a high Vt synthesis in the ovary. Two additional studies, carried out in *P. vannamei* (Quackenbush, 1989a) and in *P. semisulcatus* (Browdy *et al.*, 1990; Fainzilber *et al.*, 1992) revealed lower rates of yolk synthesis in the hepatopancreas compared to the respective ovarian rates. The rate of Vg production, normalized per mg protein, in the hepatopancreas of *P. vannamei* was around 10% of the ovarian value at early and mid vitellogenesis. An apparent increase of this percentage to 50% at late vitellogenesis of *P. vannamei*, is an artifact caused by the normalization method, as the accumulation of yolk in the ovary during vitellogenesis adds protein that does not contribute to Vt synthesis but reduces the rate of synthesis per mg protein. A further reduction of the contribution of the whole hepatopancreas *versus* the whole ovary is due to its smaller protein content. The Vg rate of synthesis in the whole hepatopancreas of *P. semisulcatus* reached 4.35% of the Vt rate of synthesis in the whole ovary. The percentages of synthesized Vg from total protein synthesis in the hepatopancreas were also low in both *P. vannamei* and *P. semisulcatus* (1 and 14.4%, respectively) compared to similar percentages in the ovary (8 and 40%, respectively).

The small contribution of the *in vitro* incubated hepatopancreas to Vg synthesis should be further established by complementary *in vivo* methods, having better resemblance to the natural vitellogenic process. An appropriate method for examination of the hepatopancreas share in yolk synthesis is the identification of Vg-mRNA in the penaeid hepatopancreas and ovary, a study yet to be done. Another approach, the one taken by the present research, includes determination of the presence of radioactively labeled Vg and Vt in the hemolymph, hepatopancreas, and the ovary of vitellogenic penaeid females following injection of labeled amino acid. It is aimed at validating the overall involvement of the hepatopancreas and the ovary (not distinguishing between production and processing of Vg/Vt) and at examining the dynamics of circulating Vg.

Materials and Methods

Animals

Adults of *Peneaus semisulcatus* were collected in Haifa Bay, Israel. Thirty shrimp were held in a 3000-liter seawater tank. Water temperature ranged from 18°C (winter) to 27°C (summer). Water was replaced at a rate of 300% per day. Animals were fed daily with defrosted *Artemia* and a mixture of defrosted shrimp, fish, and squid. Females were individually tagged by clipping their uropods. Their oogenic stage was monitored periodically by visual examination of the ovaries according to the method of Browdy and Samocha (1985). Ovarian developmental stages were determined more accurately after sacrificing the females, by measuring the average oocyte diameter (AOD) as described by Shlagman *et al.* (1986). Molting of individual females was recorded daily by identification of the clipped cods on the uropods of the collected exuviae.

Chemicals

[^{35}S]–Methionine, 300 mCi/mMol was purchased from Amersham, U.K. All other reagents were of analytical grade.

[^{35}S]–*methionine labeling and sampling of labeled tissues*

Labeled methionine was injected through the thin cuticle connecting the thorax and the abdomen using 1 ml disposable syringes. Samples of hemolymph from live individuals were collected from the same site with 1 ml syringes containing a premeasured volume of the anticoagulant 10% tri-sodium citrate. Hemolymph from sacrificed females was collected in a premeasured volume of 10% tri-sodium citrate through an excision cut in the anterior part of the cephalothorax, at the base of the eyestalk.

The ovary and hepatopancreas were dissected from each of the sacrificed females and weighed before a portion of the ovary was fixed in 4% formaldehyde in seawater for the measurement of oocyte diameters. The remaining part of the ovary was then weighed again. The hepatopancreas and ovary from each of the females were homogenized separately in 0.1 M phosphate buffer, pH 7.4. Hemolymph samples and homogenized tissues were centrifuged and the supernatants were used for the TCA precipitation and radioimmunoprecipitation (RIP).

TCA precipitation and measurement of total labeled methionine

Four 50 µl aliquots taken from each supernatant were pipetted onto Whatman 3 mm filter paper discs and dried. The proteins were precipitated onto two of the discs in 10% TCA according to Mans and Novelli (1961), and all

four discs were radioactively counted in scintillation vials with 4 ml Aquasol-2 (NEN) in a Kontron Betamatic liquid scintillation counter.

Radioimmunoprecipitation (RIP)

Radioimmunoprecipitation was carried out by mixing 25 μl samples of each supernatant with unlabeled purified vitellin (75 μg in 25 μl) and 200 μl of anti-Vt serum. The anti-Vt serum was prepared and examined for Vt specificity according to Browdy *et al.* (1990) and Tom *et al.* (1992). Control samples contained normal rabbit serum (NRS) instead of anti-Vt serum. The total volume in the reaction tube was adjusted to 0.5 ml with 0.4 M NaCl and was mixed thoroughly before overnight incubation at 4°C. Following incubation, samples were centrifuged and the pellets washed three times with 0.4 M NaCl. Finally, each pellet was dissolved in 75 μl of 0.5 M NaOH by vigorous shaking for ½ h at room temperature and was neutralized with 25 μl of 2 M HCl before it was counted.

Complete immunoprecipitation of the labeled antigen was achieved with an adequate antigen-antibody ratio. The determination of suitable amounts of reactants was carried out by reacting fixed amounts of unlabeled Vt and labeled sample with increasing amounts of anti-Vt serum, followed by washing and counting the pellets.
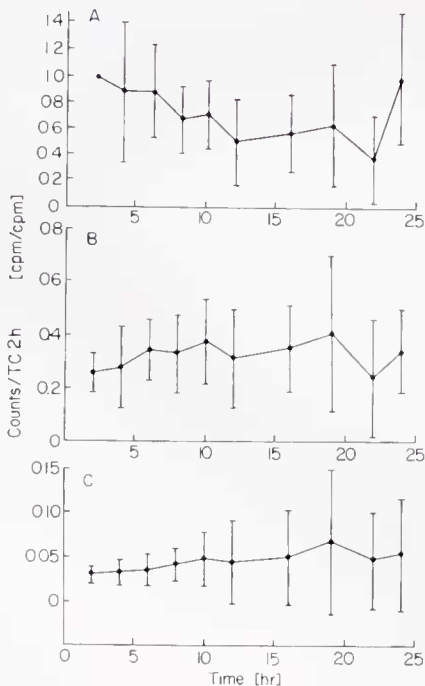

Figure 1. Twenty-four h time course presentation of (A) hemolymph total-labeled methionine, (B) labeled protein, and (C) labeled Vg normalized by the counts of total-labeled methionine in the hemolymph 2 h after injection (TC2h) in *Penaeus semisulcatus.* Vertical lines show standard deviations.

Table I

Incorporation of [^{35}S]–methionine into TCA precipitable proteins and Vg/Vt in the hepatopancreas and ovary of Penaeus semisulcatus *24 h after injection*

Parameters [*]	Ovary	Hepatopancreas
Total [^{35}S]-methionine	0.0573 ± 0.0450	0.2954 ± 0.1693
TCA precipitable protein	0.0278 ± 0.0260	0.0501 ± 0.0289
Immunoprecipitable Vt	0.0028 ± 0.0058	0.0032 ± 0.0021

[*]—dpm/total dpm in the hemolymph 2 h after injection/g dry weight of the organ (n = 11).

Results

Eleven vitellogenic females with AOD ranging from 180 to 350 μm were injected with 127 μCi of [^{35}S]-methionine dissolved in 20 μl of distilled water. The regular feeding, temperature, and water circulation regimes were maintained during the 24 h experiment. Hemolymph samples were taken every two h for the first 12 h. Samples were taken at longer intervals during the last 12 h of the incubation period to avoid stressing the sampled females. The females were sacrificed after 24 h, their hemolymph bled and their ovaries and hepatopancreas homogenized separately in a measured volume of 0.1 M phosphate buffer, pH 7.4. A piece of each ovary was taken for AOD measurement. The concentration of labeled methionine counted in the hemolymph 2 h after injection served as the normalization index, with the assumption that the injected methionine was evenly dissolved in the hemolymph following that period, as was previously found for [^{3}H]-inulin collected in the hemolymph (Shafir *et al.,* in prep.). Normalizing of protein and Vg/Vt counts by this index compensates for differences in hemolymph volume and for the amounts of injected methionine which entered the hemolymph.

Three values of radioactivity were counted in each hemolymphatic sample: total labeled methionine, labeled TCA precipitable proteins and labeled immunoprecipitated Vg (Fig. 1). There is no significant difference in the concentration of either labeled protein or Vg in the hemolymph during the entire experiment, starting from the first sampling two hours after injection. The standard deviations of the levels of labeled protein and Vg increase with time.

Incorporation of methionine into total protein and Vg/Vt in the hepatopancreas and in the ovary, 24 h after injection, is presented in Table I. The level of labeled Vg in the examined tissues could not be explained by the labeled Vg found in its hemolymphatic component alone, even if the whole tissue is considered replaced by an equal volume of hemolymph, assuming 1 g wet weight of tissue is approximately equivalent to 1 ml of hemolymph (Table II).

Table II

Average ratio (n = 11) of labeled proteins in the hepatopancreas or ovary to labeled proteins found in an identical hemolymph volume in Penaeus semisulcatus

Ratios [dpm/dpm]	TCA	RIP
Hepatopancreas/hemolymph	5.07 ± 2.13	4.08 ± 3.34
Ovary/hemolymph	4.82 ± 3.21	2.71 ± 1.33

Tissue volume was estimated by assuming 1 gm wet weight equals 1 ml of hemolymph. The hepatopancreas and the ovary were dissected 24 h after *in vivo* [^{35}S]–methionine injection. Protein labeling was determined from radioactive counting of the TCA-precipitable proteins (TCA) and the immunoprecipitated Vg/Vt (RIP).

Figure 2. Percentage of labeled Vg/Vt from the labeled proteins in the ovary (filled bars) and hepatopancreas (white bars) 24 h after [^{35}S]-methionine injection. Vertical lines show standard deviations.

The percentages of synthesized Vg/Vt from total protein synthesized in the ovary and hepatopancreas are presented in Figure 2, which shows a decrease of relative methionine incorporation into ovarian Vt as oogenesis proceeds, while the value in the hepatopancreas was constant.

Comparison of the levels of total-labeled methionine and TCA precipitable labeled protein in the examined tissues (Fig. 1a, b, and Table 1) shows that free-labeled methionine was present in the female even 24 h after injection. Free-labeled methionine was also present in the above tissues with reduced amounts of injected methionine (results not shown).

The molar ratio (P, in percents) of labeled methionine incorporated into Vg or Vt from the total methionine in the Vg or Vt was calculated using the parameters described in Table III. Molar amounts of methionine are given per gram body weight.

$$P = \frac{\text{Moles of labeled methionine}}{\text{Moles of total methionine}} \times 100$$

For the hemolymphatic Vg the result was:

$$P = \frac{28210/2.2 \times 10^6/300}{177 \times 0.03/131.2} \times 100 = 0.1056\%$$

and for the ovarian Vt the result was:

$$P = \frac{30590/2.2 \times 10^6/300}{4550 \times 0.03/131.2} \times 100 = 0.0044\%$$

The results show that 24 h after injection of labeled methionine, the ratio between labeled Vg in the hemolymph and labeled Vt in the ovary is 24 (0.1056/0.0044).

Discussion

The possible roles of the ovary, hepatopancreas and hemolymph of female penaeid shrimp in the vitellogenic process were investigated. This objective was accomplished in part by determining whether [^{35}S]-methionine had become incorporated into Vg/Vt present in these organs 24 h after its injection into the hemolymph of vitellogenic females.
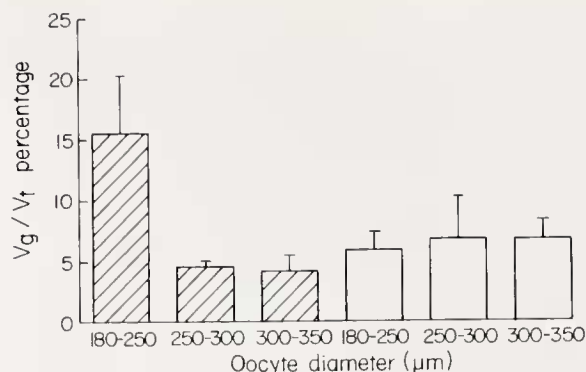
Free-labeled methionine was found in the hemolymph, ovary and hepatopancreas 24 h after injection even when an extremely small quantity of radioactive methionine was injected. This observation indicates high levels of endogeneous methionine, competing with the incorporation of labeled methionine into proteins, preventing pulse labeling of proteins. The absence of a pulse of labeled proteins and Vg did not allow determination of Vg turnover in the hemolymph. Injection of labeled Vg into vitellogenic females might be a more suitable method to achieve its pulse labeling in the hemolymph.

A relatively high percentage of *in vivo* labeled protein in the ovaries at early vitellogenesis (AOD = 180–250 μm, Fig. 2) was Vt (15.5%), which is in agreement with

Table III

Parameters used for calculating molar ratio of labeled methionine incorporated into Vg or Vt, from total methionine in Vg or Vt in Penaeus semisulcatus

Parameter	Value	Source
Percentage of methionine in Vt	3% (w/w)	Tom *et al.* (1992)
Mw of methionine residue	131.2	—
Maximum level of hemolymph Vg	177 μg/g body weight	Shafir *et al.* (in prep.)
Maximum level of ovarian Vt	4550 μg/g body weight	Shafir *et al.* (in prep.)
Specific activity of labeled methionine	300 μCi/μMol	NEN, U.S.A.
Maximum level of hemolymph labeled Vg	28210 dpm/g body weight	Present study
Maximum level of ovarian labeled Vt	30590 dpm/g body weight	Present study

the data of Shafir et ... ep.) who showed that oocytes of this size are r... mulating Vt. The reduced percentage of lab... m the labeled protein found in the presen... e vitellogenesis (4.4%, Fig. 2) when combine... slow increase of accumulated Vt in ovaries r... ame AOD range (Shafir et al., in prep.) indicates inhibition of Vt accumulation in the ovary of the intact female at AOD > 250 μm. In contrast, ovaries incubated in vitro showed no inhibition of Vt synthesis at any stage of vitellogenesis although a decrease in the percentage of synthesized Vt was observed at AOD > 350 μm, presumably due to increased synthesis of other proteins (Browdy et al., 1990).

The amount of labeled Vg and its percentage of the total labeled protein in the hepatopancreas were similar to the corresponding values of labeled Vt in the ovary after the 24 h experiment (Table 1 and Fig. 2). These labeled Vg/Vt amounts can not be explained by the hemolymphatic Vg component of the two organs alone (Table II). They are probably the result of an equilibrium between the influx of circulating Vg and the contribution of in situ produced Vg and Vg secretion during the 24 h of the experiment. These findings strongly suggest that the hepatopancreas has a more prominent role in the vitellogenic process than could be deduced from the results of earlier comparable in vitro incubations (Browdy et al., 1990; Fainzilber et al., 1992) (see also Introduction). The in vitro studies favor the penaeid ovary as the major Vt synthetic site, a conclusion supported by other studies (Yano and Chinzei, 1987; Quackenbush, 1989a). The differences between the in vivo and in vitro studies can be attributed to a more efficient Vg synthesis by the intact in vivo hepatopancreas, or to its functioning as a Vg processing site. This processing might involve addition of a polypeptide subunit which would explain the small amount of synthesis of a Vg-immunoidentical substance, or binding of a lipid component to the Vg molecule.

Absorption in penaeid ovaries of non-spawned ovulated oocytes immediately after spawning and in developing oocytes at the commencement of the premolt stage was reported by Browdy (1988). The absorption of ovulated oocytes is correlated with a relatively high Vg level in the hemolymph of recently spawned females (Shafir et al., in prep.). Consequently, secretion of Vt into the hemolymph is postulated for females with normally developing ovaries and is suggested as one explanation for the presence of Vg in the hemolymph. This Vg is assumed to be either newly synthesized Vt that will be further processed in extraovarian sites, prior to its reuptake by the developing oocytes or a degradation product of previously synthesized Vt. The 24-fold more intensive labeling of Vg compared with the Vt labeling found in the present study argues against the possibility of unselected secretion of ovarian Vt, because if this were the case, similar Vg/Vt labeling

should have been seen in both the ovary and the hemolymph.

In conclusion, evidence from this and previous studies supports the hypothesis that the hepatopancreas has a more intense involvement in the vitellogenic process than could be deduced from earlier in vitro studies, but is not necessarily the only site of Vg synthesis. The rapid accumulation of Vt that occurs early in vitellogenesis (AOD range of 150–250 μm) and the arrest of Vt accumulation later (AOD > 250 μm), first found by Shafir et al. (in prep.), is confirmed by the present findings. Furthermore, the present study shows that circulating Vg is not a degraded Vt which is secreted unselectively from the ovary.

Acknowledgments

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